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PII: S0046-8177(18)30420-9
DOI: https://doi.org/10.1016/j.humpath.2018.10.023
Reference: YHUPA 4767

To appear in: *Human Pathology*

Received date: 23 August 2018
Revised date: 15 October 2018
Accepted date: 18 October 2018

Please cite this article as: Heather Dawson, Francesca Galuppini, Peter Träger, Martin D. Berger, Peter Studer, Lukas Brügger, Inti Zlobec, Daniel Inderbitzin, Alessandro Lugli , Validation of the international tumor budding consensus conference (ITBCC 2016) recommendations on tumor budding in stage I-IV colorectal cancer. Yhupa (2018), https://doi.org/10.1016/j.humpath.2018.10.023

Validation of the International Tumor Budding Consensus Conference (ITBCC 2016) recommendations on tumor budding in Stage I-IV colorectal cancer

Heather Dawson MD[a*], Francesca Galuppini MD[b*], Peter Träger[c], Martin D. Berger MD[d], Peter Studer MD, PhD[e], Lukas Brügger MD[e], Inti Zlobec PhD[a], Daniel Inderbitzin MD[f], Alessandro Lugli MD[a]

*equally contributing first authors

[a] Institute of Pathology, University of Bern, Switzerland
[b] Pathology Unit, Department of Medicine (DIMED), University of Padova, Italy
[c] Careanaesth AG, Zurich, Switzerland
[d] Department of Medical Oncology, Inselspital, Bern University Hospital, University of Bern, Switzerland
[e] Clinic of Visceral Surgery, Inselspital, Bern University Hospital, University of Bern, Switzerland
[f] Department of General Surgery and Visceral Surgery, Bürgerspital Solothurn, Switzerland

Keywords: Tumor budding, colorectal cancer, consensus conference

Running title: Tumor budding in Stage I-IV colorectal cancer

Declarations of interest: None
Funding disclosures: This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Corresponding Author:

Heather Dawson, MD
Institute of Pathology, University of Bern
Murtenstrasse 31, P.O. Box 62,
CH-3008 Bern
Switzerland
Tel. +41 31 632 9960
Fax +41 31 632 0969
heather.dawson@pathology.unibe.ch

Abstract (max. 250 words):

Tumor budding is a robust prognostic parameter in colorectal cancer and can be used as an additional factor to guide patient management. Though backed by large bodies of data, a standardized scoring method is essential for integrating tumor budding in reporting protocols. The International Tumor Budding Consensus Conference (ITBCC) 2016 has proposed such a scoring system. The aim of this study is to validate the ITBCC method of tumor budding assessment on a well-characterized CRC cohort. 379 patients with resected Stage I-IV colorectal cancer were entered into the study. Tumor budding was scored by two pathologists according to the ITBCC recommendations on hematoxylin and eosin-stained slides and scored as BD1 (low-), BD2 (intermediate-) and BD3 (high-grade). Analysis was performed using a 3-tier approach, a 2-tier approach (BD1+2 versus BD3) and budding as a continuous variable. High-grade tumor budding was associated with adverse clinicopathological features including higher pT, higher pN stage and higher TNM stage (all $p < 0.001$) and poorer overall survival on univariate analysis ($p=0.0251$ for BD1/2/3, $p=0.0106$ for BD1+2 versus BD3 and $p=0.0195$ for continuous scores (HR 1.023 (95%CI: 1.004-1.043 per bud)). In stage II cancers, BD3 was associated with poorer disease-free survival ($p<0.01$). Tumor budding assessed by the method proposed by the ITBCC is applicable to colorectal cancer resection specimens and can be used for widespread reporting in routine.

1. Introduction

In colorectal cancer (CRC), TNM staging is the most important factor in determining prognosis and patient management [1]. However, a wide biological heterogeneity of individual tumors may account for considerable differences in tumor behavior seen in patients within the same stage. Therefore, additional biomarkers to better stratify outcome are sought after within certain patient groups. In the avid search for such potential biomarkers in CRC, tumor budding has emerged as an especially robust and promising candidate.

Tumor budding, defined as single tumor cells or small clusters of ≤4 tumor cells at the invasive front of CRC [2], is a morphologically visible sign of tumor dissemination and has been linked to epithelial-to-mesenchymal (EMT)-like processes [3]. Most recently, transcriptome profiling studies have linked tumor buds with the mesenchymal type (Consensus Molecular Classification type 4, CMS 4) of CRC [4]. It is therefore not surprising that large bodies of data have consistently demonstrated tumor budding to be an independent adverse prognostic marker in CRC and associated with unfavourable clinico-pathological features, nodal and distant metastases [5-7].

Due to its potential clinical implications, tumor budding has been especially well studied in two scenarios. In endoscopically resected pT1 CRC, tumor budding has been demonstrated to be a strong and independent predictor of nodal metastases, and may help select patients for segmental resection with lymphadenectomy [2, 8-11]. In stage II CRC patients, high-grade budding tumors have been shown to behave aggressively [2, 12] and similarly to stage III CRC [13-15], with shorter survival times and higher rates of recurrence; therefore, these patients may be offered adjuvant chemotherapy.

Although the data in the literature would certainly support the integration of tumor budding in reporting protocols, the lack of a standardized scoring system has been one of the major barriers to

the routine reporting of tumor budding in CRC. The objective of the International Tumor Budding Consensus Conference (ITBCC) was to provide an evidence-based set of recommendations for such a standardized method [2]. Since published, the ITBCC guidelines have been included as an additional reporting parameter in the protocol of the College of American Pathologists (CAP) with the recommendation to report tumor budding in pT1 and stage II CRC [16].

While largely based on the method proposed by Ueno et al [10], further validation studies should provide the basis for more solid implementation of the ITBCC method in CRC reporting. Therefore, the aim of this study was to validate the ITBCC method of tumor budding assessment in a large, well-characterized CRC cohort.

2. Materials and methods

2.1. Patient cohort

379 patients with primary CRC resected between 2002 and 2014 were entered into this study. Haematoxylin and eosin (H&E)-stained slides were re-reviewed by two expert gastrointestinal pathologists (A.L., H.D.) according to the UICC TNM 7th edition [1] for pathological features. In addition to standard reporting elements, this included assessment of peritumoral inflammation (Klintrup-Mäkinen score) and tumor border configuration. The Klintrup-Mäkinen score was obtained by evaluating the overall inflammatory reaction at the invasive margin on a four-degree scale ranging from 0 (no increase of inflammatory cells) to 3 (very prominent inflammatory reaction with invariable and frequent destruction of cancer cell islets) [17]. Tumor border configuration was assessed according to Karamitopoulou et al [18] by scoring the percentage of infiltrating tumor margin in 5% increments. Tumors were categorized as either right (caecum, ascending colon, hepatic flexure and transverse colon), left (splenic flexure, descending colon, sigmoid) or rectal based on surgical and pathological reports. Exclusion criteria included pre-operative chemoradiotherapy, previous endoscopic resection, tumors with pure signet ring cell and mucinous histology in accordance with

the ITBCC guidelines and patients with time of death within 1 month after surgery. Patient follow-up was scheduled according to the recommendations of the Swiss Society of Gastroenterology for surgically resected colorectal tumors [19]. Follow-up data including overall survival (OS) time (available for all patients, median 127.6 months, 95%CI: 99-147) and disease free survival (DFS) time (available for 78 patients in subset analysis of stage II patients, mean 37.3 months) was obtained. Ethics approval was granted by the Cantonal Ethics Commission of Bern (KEK 2017-01803, Oct. 24, 2017).

2.2 Assessment of tumor budding

Tumor budding was scored by two observers (H.D, and F.G.) according to the ITBCC 2016 recommendations [2]: H&E-stained sections were scanned at medium power (10x) to identify the densest area of budding at the tumor front ('hotspot'). Tumor buds were counted in this area at 20x magnification (Nikon Eclipse E600, 750 objective, field diameter of 0.25 mm, area of 1.227 mm2; Nikon AG Instruments, Egg, Switzerland). The bud count was divided by the normalization factor (1.563) relative to the specific microscope eyepiece field number (FN) diameter to determine the tumor bud count per 0.785mm2. The final bud count and the budding category (Bd 1: 0-4 buds, Bd2: 5-9 buds, Bd3: 10 buds or more, Fig. 1) were recorded. Cases with pure mucinous or signet ring cell morphology were excluded from analysis. Selected cases were discussed at a multiheader microscope to obtain a consensus score.

2.3 Mismatch repair protein (MMR) status

Immunohistochemistry for mismatch repair (MMR) proteins MLH1, MSH2, MSH6 and PMS2 was available for 342 patients, and performed as previously described [20]. Markers were considered positive if any nuclear expression in tumor cells was seen. Tumors negative for at least one marker were considered MMR-deficient, and MMR-proficient if all markers were expressed in the tumor.

Since information on family history was unavailable, no attempt was made to further subdivide patients into Lynch syndrome or sporadic MSI.

2.4 Statistical analysis

Descriptive statistics for categorical and continuous variables were analysed. The differences between categorical histopathological features and BD categories was carried out using the Chi-Square test. The association with continuous budding counts was analysed with the Wilcoxon Rank Sum test. For age and percentage of expanding tumor border configuration, the Pearson correlation analysis was performed. Log rank test and Kaplan-Meier method were used for univariate survival time analysis. OS and DFS were the endpoints of interest. Multivariate survival analysis was performed using Cox regression analysis. Hazard ratios (HR) and 95%CI were used to determine effect size. P-values were all two-sided and considered significant when $p<0.05$.

3. Results

3.1 Associations of tumor budding with clinicopathological features and survival

Across all stages (n=379), tumor budding was low-grade (BD1) in 149 patients, intermediate-grade (BD2) 101 patients and high-grade (BD3) in 129 patients (illustrated in Fig 1A-D). Frequencies of each variable and associations with BD category (BD1, BD2 and BD3 separately), a 2-tier classification (BD1+2 vs. BD3) and continuous scores are included in Table 1. As expected, higher tumor budding grades were seen in patients having received post-operative therapy (BD1/2/3: p=0.032, BD1/2 vs. BD3: 0.0016 and 0.0044 for continuous scores, respectively), patients with higher pT-stage (<0.001, all), nodal metastases (p<0.001, all), more advanced TNM stage (p<0.001, all), higher tumor grade (BD1/2/3: p=0.002, BD1/2 vs. BD3: p=0.002 and p<0.001 for continuous scores, respectively), lymphatic invasion (p<0.001, all), venous invasion (p<0.001, all), perineural invasion (p<0.001, all). Higher tumor budding was inversely associated with expanding tumor border configuration (p<0.001,

all) and higher peritumoral inflammation (Klintrup-Mäkinen score; p=0.026, p=0.0048 and p=0.0258, respectively). No significant differences in BD scores were seen in different tumor sites and in MMR proficient vs deficient tumors.

On univariate analysis, tumor budding was significantly associated with shorter overall survival times in a 3-tier (BD1 vs. BD2 vs. BD3, p=0.0251, Fig. 2A) and 2-tier approach (BD1-2 vs. BD3, p=0.0106, Fig. 2B) and when taken as a continuous variable (HR 1.023 (95% CI: 1.004-1.043) per increased bud, p=0.0195). However, significant associations between tumor budding and survival were lost in multivariate analysis (Table 2) including BD category (p=0.0911), TNM stage (stage IV: p=0.0041) and postoperative therapy (p=0.1022).

3.2 Analysis of tumor budding, clinicopathological features and survival in Stage II patients

In stage II patients (n=109,) tumor budding was low-grade (BD1) in 54 (49.5%) patients, intermediate-grade (BD2) in 31 (28.4%) patients and high-grade (BD3) in 24 (22.0%) patients. Frequencies of each variable and associations with BD category (BD1, BD2 and BD3 separately), a 2-tier classification (BD1+2 vs. BD3) and continuous scores can be found in Table 3. In univariate analysis, tumor budding was not associated with overall survival time but with 5-year disease-free survival (p=0.0084, Fig 2C). As only 11 patients received post-operative therapy, no conclusions regarding possible associations between tumor budding grade, postoperative therapy and survival could be made.

4. Discussion

In this study, we provide a validation of the ITBCC scoring method on a large, well-characterized mixed-stage CRC cohort. Assessed by this method and in line with previous results in the literature using similar and other scoring systems, tumor budding was associated with adverse clinico-pathological features and survival both in the entire cohort and in a subset of stage II patients.

Although several different tumor budding scoring methods have been proposed in the literature, the ITBCC consensus scoring method is largely based on the system proposed by Ueno et al [10]. Key aspects of this proposal (hot-spot, 20x magnification and 0,785 mm2) were adopted by the Japanese Society for Cancer of the Colon and the Rectum (JSCCR) prior to the ITBCC, and routine reporting of tumor budding in pT1 tumors according to this method has been performed since 2009 [21] . Indeed, this system has been used in studies including large Japanese patient cohorts with pT1 CRC [11, 22] and by others with slight variations, such as an adapted HPF size of 0.95mm^2 [23-25]. However, a standardized consensus approach such as provided by the ITBCC guidelines is essential for tumor budding to be validated, to compare study results and ultimately be used as a biomarker in routine diagnostics.

One important aspect of the ITBCC recommendations is the separate reporting of tumor budding grade (BD 1,2 or 3) and the raw tumor bud count. As a numerical variable on a biological spectrum, continuous tumor bud counts are expected to provide more precise risk stratification than cut-offs alone (as seen in Table 1). It must also be emphasized that relevant cut-offs will vary according to the clinical endpoint of a certain scenario (nodal metastases for pT1 tumors where BD2 and BD3 are considered high-risk; and recurrence/survival in advanced tumors where only BD3 is considered a risk factor) [2]. Hence, a two-tier classification (BD1+2 vs. BD3) was preferred to better stratify patients in this cohort, where pT1 tumors were underrepresented (n=30) and therefore not analyzed separately. In this study using a mixed stage cohort including patients with distant metastases, tumor budding was not an independent prognostic factor on multivariate analysis including TNM stage. This expected result emphasizes the importance and relevance of accurate clinical and pathological staging in routine as a basis for complementary biomarker studies. In a separate analysis of stage II patients, tumor budding was significantly associated with shorter 5 year DFS but not with shorter OS. This finding must be interpreted with caution as only few events were observed in this relatively small patient group, but underlines DFS as a more meaningful survival parameter in stage II tumors.

Tumor budding in CRC is a robust biomarker which is simple to use and can be assessed using routine light microscopy on H&E stained slides. The standardized method as proposed by the ITBCC consensus recommendations will hopefully pave the way of integrating tumor budding in reporting protocols as has recently been the case for the latest update of the CAP CRC checklist. Further validation studies such as we present here aim to promote more widespread integration of the ITBCC consensus method in standardized reporting of tumor budding in CRC.

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Figure legends

Figure 1: Tumor budding as assessed by the ITBCC 2016 guidelines. (A) No tumor budding and (B) low-grade tumor budding (both BD1, 0-4 tumor buds/0.785mm^2). (C) Intermediate-grade tumor budding (BD2, 5-9 tumor buds/0.785mm^2). (D) High-grade tumor budding (BD3, ≥10 tumor buds/0.785mm^2). Arrows point to selected tumor buds in all images.

Figure 2: Survival curves of CRC patients stratified by budding status. (A) Overall survival in all patients (n=379) with low- (BD1), intermediate- (BD2) and high-grade budding (BD3). (B) Overall survival in all patients (n=379) stratified by BD1+2 versus BD3. (C) Disease-free survival in Stage II patients (n=78) stratified by BD1+2 versus BD3.

Table 1: Patient characteristics (n=379) and association of tumor budding (BD category) with clinicopathological features

Features		Frequency N (%)	BD Category			P-value		P-value
			BD1	BD2	BD3	BD1,2,3	BD1+2 vs 3	Continuous score
Age (yrs) (n=379)	Mean	69.6	70.0	69.3	69.4	0.9714	0.9008	0.4777
Gender (n=379)								
	Female	150 (39.6)	59 (39.6)	32 (31.7)	59 (45.7)	0.0964	0.0782	0.4839
	Male	229 (60.4)	90 (60.4)	69 (68.3)	70 (54.3)			
Histological subtype (n=378)								
	Adenocarcinoma	319 (84.4)	121 (81.8)	86 (85.2)	112 (86.8)	0.5738	0.6446	0.3979
	Mucinous	52 (13.8)	25 (16.9)	12 (11.9)	15 (11.6)			
	Other	7 (1.9)	2 (1.4)	3 (3.0)	2 (1.6)			
Tumor location (n=379)								
	Left	229 (60.4)	91 (61.1)	60 (59.4)	78 (60.5)	0.9655	0.9902	0.6809
	Right	150 (39.6)	58 (38.9)	41 (40.6)	51 (39.5)			
Post-operative therapy (n=232)								
	None	172 (74.1)	81 (84.4)	36 (75.0)	55 (62.5)	0.0032[a]	0.0016[a]	0.0004[a]
	Treated	60 (25.9)	15 (15.6)	12 (25.0)	33 (37.5)			
pT (n=378)								
	pT1	30 (7.9)	20(13.4)	6 (5.9)	4 (3.1)	<0.0001[a]	<0.0001[a]	<0.0001[a]
	pT2	47 (12.4)	23 (15.4)	14 (13.9)	10 (7.8)			
	pT3	190 (50.1)	84 (56.4)	49 (48.5)	57 (44.2)			
	pT4	111 (29.3)	21 (14.2)	32 (31.7)	58 (45.0)			
pN (n=370)								
	pN0	185 (50.0)	98 (67.1)	52 (52.5)	35 (28.0)	<0.0001[a]	<0.0001[a]	<0.0001[a]
	pN1	115 (31.1)	38 (26.0)	35 (35.4)	42 (33.6)			
	pN2	70 (18.9)	10 (6.9)	12 (12.1)	48 (38.4)			
TNM stage (n=347)								
	I	57 (16.4)	34 (24.8)	16 (17.2)	7 (6.0)	<0.0001[a]	<0.0001[a]	<0.0001[a]
	II	109 (31.4)	54 (39.4)	31 (33.3)	24 (20.5)			
	III	111 (32.0)	37 (27.0)	32 (34.4)	42 (35.9)			
	IV	70 (20.2)	12 (8.8)	14 (15.1)	44 (37.6)			
Tumor grade								

(n=364)								
	G1	21 (5.8)	16 (11.4)	5 (5.0)	0 (0.0)	0.002 [a]	0.002 [a]	<0.0001 [a]
	G2	264 (72.5)	97 (68.8)	76 (76.0)	91 (74.0)			
	G3	79 (21.7)	28 (19.9)	19 (19.0)	32 (26.0)			
Lymphatic invasion (n=362)								
	L0	146 (40.3)	94 (65.7)	36 (38.3)	16 (12.8)	<0.0001 [a]	<0.0001 [a]	<0.0001 [a]
	L1	216 (59.7)	49 (34.3)	58 (61.7)	109 (87.2)			
Venous invasion (n=365)								
	V0	213 (58.4)	107 (74.3)	56 (58.3)	50 (40.0)	<0.0001 [a]	<0.0001 [a]	<0.0001 [a]
	V1	152 (41.6)	37 (25.7)	40 (41.7)	75 (60.0)			
Perineural invasion (n=346)								
	Pn0	276 (79.8)	127 (93.4)	78 (85.7)	71 (59.7)	<0.0001 [a]	<0.0001 [a]	<0.0001 [a]
	Pn1	70 (20.2)	9 (6.6)	13 (14.3)	48 (40.3)			
MMR status (n=258)								
	MMR-deficient	36 (14.0)	10 (10.9)	11 (14.9)	15 (16.3)	0.5479	0.4172	0.3876
	MMR-proficient	222 (86.1)	82 (89.1)	63 (85.1)	77 (83.7)			
Klintrup-Mäkinen (n=304)								
	0	15 (4.9)	5 (3.9)	1 (1.3)	9 (9.0)	0.0206 [a]	0.0048 [a]	0.0258 [a]
	1	137 (45.1)	61 (48.0)	37 (48.1)	39 (39.0)			
	2	116 (38.2)	45 (35.4)	25 (32.5)	46 (46.0)			
	3	36 (11.8)	16 (12.6)	14 (18.2)	6 (6.0)			
Expanding tumor border (%) (n=298)	Mean	46.5	61.5	42.4	30.9	<0.0001 [a]	<0.0001 [a]	<0.0001
Overall survival time (months) (n=379)	Median (95%CI)	127.6 (99-147)	103.2+-5.7	93.1+-6.6	67+-4.2	0.0251 [a]	0.0106 [a]	0.0195 [a.b]

[a] Statistically significant values (p<0.05). [b]Increased HR per bud: 1.023 (95%CI: 1.004-1.043). Abbreviations: mismatch repair (MMR)

Table 2: Multivariate analysis of tumor budding (BD) category along with TNM stage and postoperative therapy

Feature		HR (95%CI)	P-value
BD category	BD1+2	1.0	0.0911
	BD3	1.51 (0.94-2.42)	
TNM stage	IV	1.0	0.0041
	I	0.23 (0.08-0.62)	
	II	0.62 (0.33-1.15)	0.127
	III	0.75 (0.43-1.3)	0.3214
Postoperative therapy	None	1.0	0.1022
	Treated	0.65 (0.38-1.09)	

Table 3: Association of tumor budding (BD category) with clinicopathological features in stage II patients (n=109)

Features		Frequency N (%)	BD Category			P-value		P-value
			BD1	BD2	BD3	BD1,2,3	BD1+2 vs 3	Continuous score
Age (yrs) (n=109)	Mean	69.5	69.5	69.0	69.8	0.9471	0.7755	0.5387
Gender (n=109)	Female	48 (44.0)	23 (43.6)	11 (35.5)	14 (58.3)	0.2281	0.1101	0.422
	Male	61 (56.0)	31 (57.4)	20 (64.5)	10 (41.7)			
Histological subtype (n=109)	Adenocarcinoma	89 (81.7)	44 (81.5)	25 (80.7)	20 (83.3)	0.6262	0.8593	0.5759
	Mucinous	19 (17.4)	10 (18.5)	5 (16.1)	4 (16.7)			
	Other	1 (0.9)	0 (0.0)	1 (3.2)	0 (0.0)			
Tumor location (n=109)	Left	56 (51.4)	33 (61.1)	12 (38.7)	11 (45.8)	0.1145	0.5384	0.1633
	Right	53 (48.6)	21 (38.9)	19 (61.3)	13 (54.2)			
Post-operative therapy (n=68)	None	57 (83.8)	32 (88.9)	13 (81.3)	12 (75.0)	0.4321	0.2731	0.1723
	Treated	11 (16.2)	4 (11.1)	3 (18.8)	4 (25.0)			
pT (n=109)	pT3	88 (80.7)	48 (88.9)	24 (77.4)	16 (66.7)	0.0614	0.0478	0.0846
	pT4	21 (19.3)	6 (11.1)	7 (22.6)	8 (33.3)			
Tumor grade (n=105)	G1	6 (5.7)	4 (8.0)	2 (6.5)	0 (0.0)	0.1851	0.0497	0.5626
	G2	83 (79.1)	41 (82.0)	25 (80.7)	17 (70.8)			
	G3	16 (15.2)	5 (10.0)	4 (12.9)	7 (29.2)			
Lymphatic invasion (n=103)	L0	69 (67.0)	41 (82.0)	19 (63.3)	9 (39.1)	0.0013	0.0013	0.0003
	L1	34 (33.0)	9 (18.0)	11 (36.7)	14 (60.9)			
Venous invasion (n=104)	V0	79 (76.0)	44 (86.3)	19 (63.3)	16 (69.6)	0.0472	0.416	0.0572
	V1	25 (24.0)	7 (13.7)	11 (36.7)	7 (30.4)			
Perineural invasion (n=99)	Pn0	90 (90.9)	47 (97.9)	27 (93.1)	16 (72.7)	0.0027	0.0008	0.0023

	Pn1	9 (9.1)	1 (2.1)	2 (6.9)	6 (27.3)			
MMR status (n=66)	MMR-deficient	12 (18.2)	3 (9.7)	5 (27.8)	4 (23.5)	0.229	0.507	0.1581
	MMR-proficient	54 (81.8)	28 (90.3)	13 (72.2)	13 (76.5)			
Klintrup-Mäkinen (n=89)	0	2 (2.3)	0 (0.0)	0 (0.0)	2 (10.5)	0.1818	0.0429	0.0306
	1	38 (42.7)	22 (47.8)	9 (37.5)	7 (36.8)			
	2	40 (45.0)	19 (41.3)	12 (50.0)	9 (47.4)			
	3	9 (10.1)	5 (10.9)	3 (12.5)	1 (5.3)			
Expanding tumor border (%) (n=109)	Mean	56.7	67.4	42.8	47.3	0.0023	0.1526	<0.0001

Abbreviations: mismatch repair (MMR)

Highlights:

- The ITBCC 2016 has initiated the inclusion of tumor budding in CRC reporting protocols
- Validation studies provide further ground to implement the ITBCC scoring system in routine
- ITBCC tumor budding is recommended as an additional prognostic factor in CRC

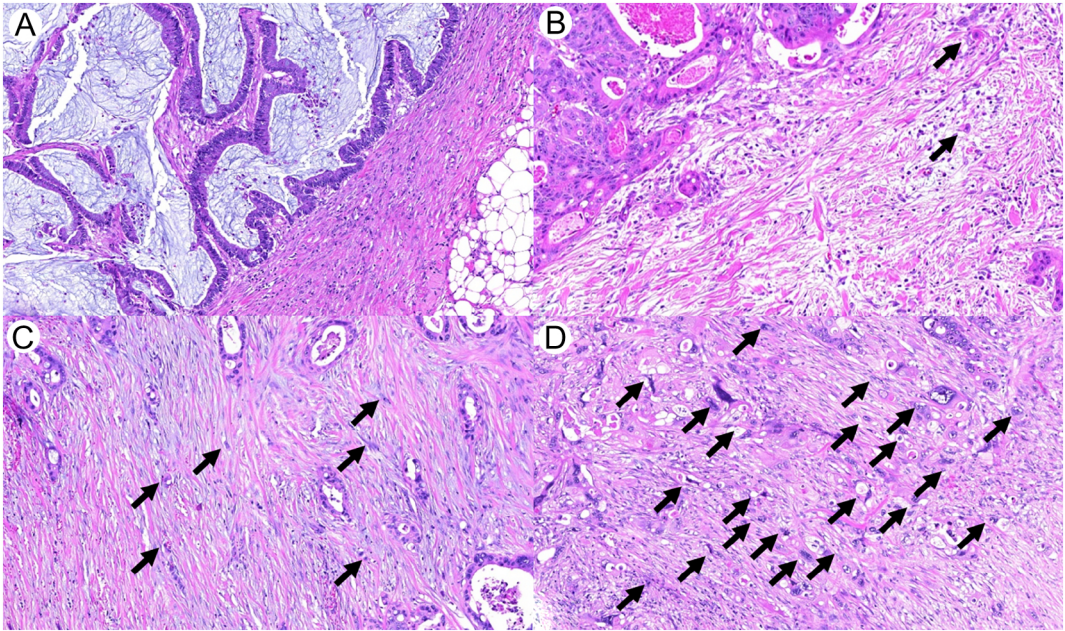

Figure 1

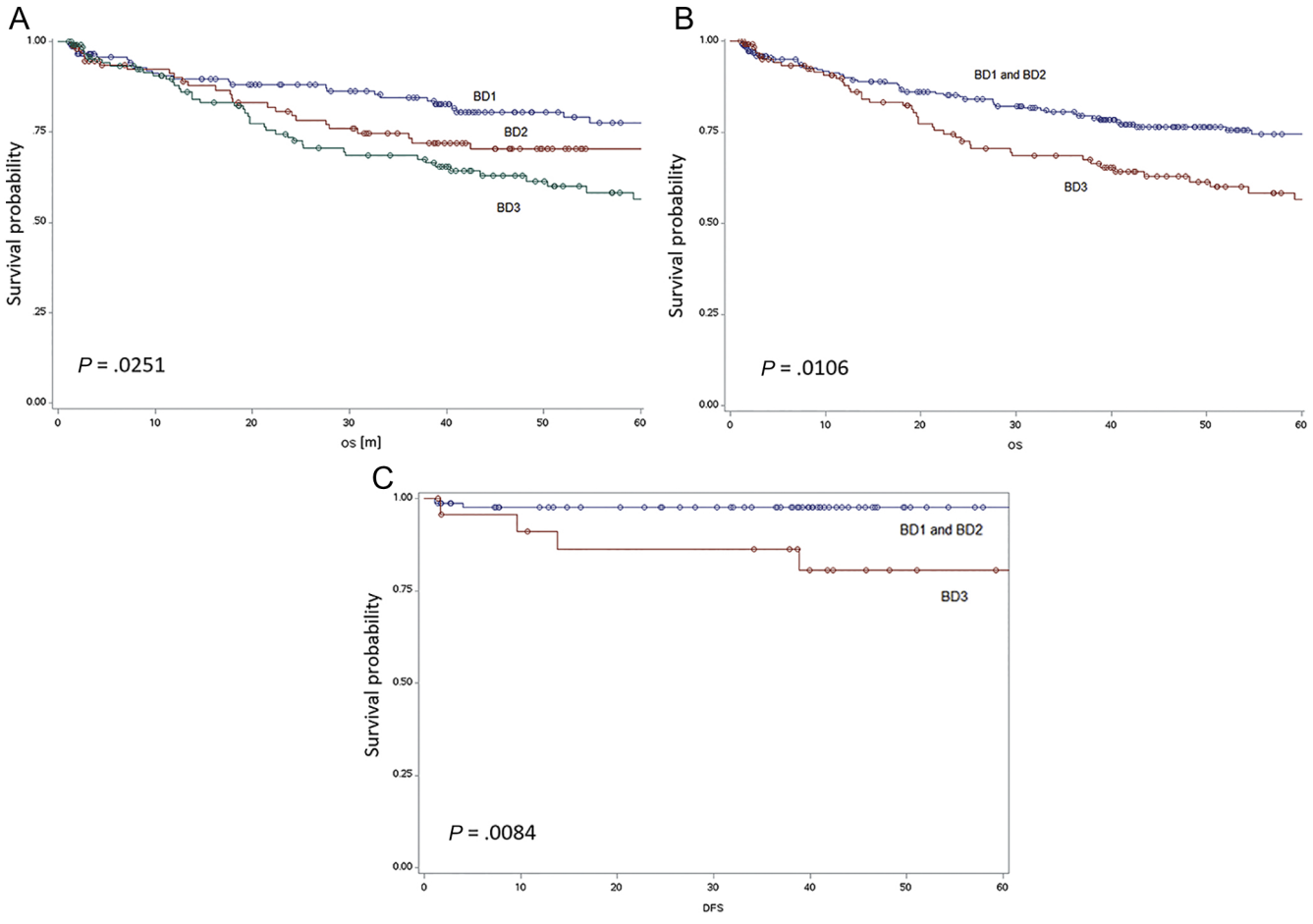
A
Survival probability
1.00
.75
.50
.25
0.00
BD1
BD2
BD3
P = .0251
0
10
20
30
40
50
60
os [m]
B
Survival probability
1.00
0.75
0.50
0.25
0.00
BD1 and BD2
BD3
P = .0106
0
10
20
30
40
50
60
OS
C
Survival probability
1.00
0.75
0.50
0.25
0.00
BD1 and BD2
BD3
P = .0084
0
10
20
30
40
50
60
DFS

Figure 2